Characterization of a differentially expressed protein that shows an unusual localization to intracellular membranes in *Leishmania major*

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The *SHERP* genes are found as a tandem pair within the differentially regulated *LmcDNA16* locus of *Leishmania major*. The *SHERP* gene product (small hydrophilic endoplasmic reticulum-associated protein) is unusual in its small size (6.2 kDa), its acidic pI (4.6) and its exclusive, high-level expression ($\approx$ 100000 copies per cell) in infective non-replicative parasite stages. No homologues have been found to date. Secondary-structure predictions suggest that SHERP contains an amphiphilic α-helix that is presumably involved in protein–protein interactions. SHERP has been localized to the endoplasmic reticulum as well as to the outer mitochondrial membrane in both wild-type and over-expressing parasites. Given the absence of an N-terminal signal sequence, transmembrane-spanning domains or detectable post-translational modifications, it is likely that this hydrophilic molecule is a peripheral membrane protein on the cytosolic face of intracellular membranes. This weak membrane association has been confirmed in cell-fractionation assays, in which SHERP redistributes from the cytoplasmic to the membrane fraction after *in vivo* cross-linking. SHERP does not appear to be involved in rearrangements of the cytoskeleton or conservation of organelle morphology during parasite differentiation. The role of this novel protein, presumed to be part of a protein complex, in infective parasites that are nutrient-deficient and pre-adapted for intracellular survival in the mammalian host is under investigation.

Key words: differential gene expression, endoplasmic reticulum, metacyclic, mitochondrial protein.

INTRODUCTION

Flagellated protozoan parasites of the genus *Leishmania* are causative agents of a spectrum of diseases, termed the leishmaniases. Worldwide, 12 million individuals are infected, predominantly in developing countries, and 2 million new cases are diagnosed per year, of which 500000 are visceral leishmaniasis that can be fatal if left untreated (http://www.who.int/inf-fs/en/fact116.html).

Leishmania live as motile extracellular procyclics in the midgut of their dipteran vector, the sandfly, but differentiate into non-replicative, infective metacyclics prior to transmission to the mammalian host. Once inoculated, the metacyclics are phagocytosed by macrophages, in which they transform into non-motile, intracellular, replicative amastigotes and become resident within one or more specialized parasitophorous vacuoles [1].

The differentiation from non-infective procyclics into infective metacyclic parasites (metacyclogenesis) is a prerequisite for resistance to complement-mediated lysis as well as intracellular survival [2,3]. *In vitro*, metacyclic differentiation correlates with stationary phase growth and is characterized by cessation of cell division and a general down-regulation of synthetic activity [4,5]. Few molecules have so far been described that characterize the biochemically and morphologically distinct metacyclics. Of these, lipophosphoglycan, the major surface glycoconjugate, is the best studied. Lipophosphoglycan undergoes species-specific modifications during metacyclogenesis with an increase in repeat units causing an extended glycocalyx, which protects metacyclics from complement-mediated lysis in the mammalian host [6].

A number of genes have also been identified that are up-regulated in metacyclic parasites. Among these are the *gp63* genes of several species, which code for the abundant surface zinc metalloproteinase [7] and the cysteine proteinase genes of *L. mexicana* [8]. However, these genes have functional homologues in other life-cycle stages. By contrast, differential cDNA library screening [9] has led to the characterization of two metacyclic-specific proteins. The first of these, a novel 11.5 kDa protein expressed predominantly in metacyclics, is encoded by the single-copy *meta* 1 gene and localizes to vesicles near the flagellar pocket, the principal site of parasite exocytosis and endocytosis [10,11].

The second metacyclic protein, described in this paper, is SHERP [small hydrophilic endoplasmic reticulum (ER)-associated protein]. A tandem pair of *SHERP* genes is found within the *L. major LmcDNA16* gene family, which also contains three differentially expressed *HASP* genes (encoding hydrophilic acylated surface proteins) [12,13]. SHERP has a number of distinct characteristics and is expressed only in metacyclics, in which it shows weak association with the ER and outer mitochondrial membrane. This dual intracellular location for a non-integral membrane protein is unusual in eukaryotic cells and may suggest novel functions *in vivo*.

EXPERIMENTAL

Parasites

Parasites were maintained on slopes of biphasic rabbit-blood agar NNN medium at 23 °C and subcultured at 26 °C as described

Abbreviations used: SHERP, small hydrophilic endoplasmic reticulum-associated protein; DAPI, 4′,6′-diamidino-2-phenylindole; ER, endoplasmic reticulum; ORF, open reading frame; UTR, untranslated region; BiP, immunoglobulin heavy-chain binding protein; HASP, hydrophilic acylated surface protein.

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previously [9]. Strains used were *L. major* FVI (MHOM/IL/80/Friedlin) and the *LmcDNA16* double-knockout clone, Δ*cDNA16*::*HYG*/Δ*cDNA16*::*PAC* (P. G. McKean, P. W. Denny, E. Knuepfer and D. F. Smith, unpublished work; details available from the corresponding author on request, e-mail d.smith@ic.ac.uk).

Parasite RNA analysis

Total RNA was prepared from 1×10^8 parasites using Tri Reagent™ LS (Sigma), denatured with formamide/formaldehyde, separated on a 1.2% (w/v) formaldehyde/agarose gel and blotted on to Hybond-N membrane (Amersham Pharmacia Biotech) using high-salt buffer. Radioactive probes were generated by random-primed oligonucleotide labelling. Hybridization was performed in $6 \times$ SSC (where $1 \times$ SSC is 0.15 M NaCl/0.015 M sodium citrate)/$5 \times$ Denhardt's/0.5% (w/v) SDS/200 μg/ml heat-denatured herring sperm DNA at 65 °C for 12–36 h.

Cloning, expression and analysis of SHERP

A DNA fragment encoding the most 5′ open reading frame (ORF) in the *SHERP* genes was amplified from plasmid p2A [13a] using the oligonucleotides DA-PET1 (5′-GAGTCAGCTAGCATGGACCAGGAGACAAGGGACCAG-3′) and DA-PET2 (5′-TATCGGCTCGAGCGAGCCACCGCTTAGCTTGTCCTT-3′). The PCR product was cloned into pGEM-T vector (Promega), sequenced, digested with *Nhe*I and *Xho*I and cloned subsequently into the expression vector pET33b(+) (Novagen), generating pDA-PET 33/7. The junction points and insertion were confirmed by sequencing.

pDA-PET 33/7 was transformed into *Escherichia coli* BL21 (DE3) pLysS and expression of recombinant protein rSHERP-2xHis (containing N- and C-terminal histidine tags) was induced with 1 mM isopropyl-β-D-thiogalactoside for 3 h at 37 °C in SOC medium [13b]. rSHERP-2xHis was purified using Ni^{2+}-nitrilotriacetate affinity chromatography (Qiagen). A single N-terminal His-tagged version of SHERP was additionally generated using the following primers for amplification: DA-PET3 (5′- CTGAGTCCCATATGGACCAGGAGACAAGGGACCAGATG-3′) and DA-PET4 (5′-GGTATCGGATCCTTACGAGCCACCGCTTAGCTTGTC-3′). After cloning into pGEM-T vector, the construct was digested with *Nde*I and *Bam*HI and cloned into the expression vector pET15b (Novagen). Expression and purification of the recombinant protein rSHERP-His was carried out as described above.

Protein signal sequence, transmembrane-region predictions and motif searches were carried out using the PSORT II server at http://psort.nibb.ac.jp. Secondary-structure predictions were made using the PHD prediction program available at the Predict Protein server at http://www.embl-heidelberg.de. Helical-wheel and sequence alignments were carried out using the following web servers: http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html and http://www2.ebi.ac.uk/clustalw/.

Purification of antibodies specific for SHERP

Small-scale elution of antibodies from PVDF-membrane strips of immunoblots carrying purified rSHERP-2xHis protein was carried out as described previously [14], to produce highly purified antibody in low concentrations (anti-rHisD antibody) for use in immunocytochemistry. For large-scale purification of anti-SHERP antibody, rSHERP-2xHis protein was coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech). Rabbit antiserum raised against rSHERP-2xHis protein was affinity-purified and concentrated [15].

Transfection of His-tagged SHERP constructs into *L. major* FVI and Δ*cDNA16*::*HYG*/Δ*cDNA16*::*PAC*

The first *SHERP* ORF was amplified from plasmid 2A using the primers DA-PX1 (5′-GAGTCAGGATCCATCGATCCGCGCGGACCAAGATGGACCAGGAGACAAGGGACCAG-3′) and DA-PX2 (5′-TATCGGGCGGCCGCTTAGCTGCCGCGCGGCACCAGGCCGCTGCTGTGGTGGTGGTGGTGGTGGCTGCTGCCCGAGCCACCGCTTAGCTTGTCCTT-3′). The DA-PX2 primer contains a *Not*I restriction site and encodes an 18 amino acid motif including a hexahistidine motif (Gly-Ser-Ser-His-His-His-His-His-His-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser), which is recognized by rabbit polyclonal IgG antibody sc-G18 (Santa Cruz Biotechnology). The PCR product was cloned into pGEM-T vector, sequenced, digested with *Bam*HI/*Sal*I and subcloned into pTEX vector [16], generating the [*pTEX NEO SHERP*^{His}] plasmid. Transfections into *L. major* FVI parasites and *LmcDNA16* double-knockout parasites (Δ*cDNA16*::*HYG*/Δ*cDNA16*::*PAC*) were performed as described previously [17]. Cultures were subsequently grown in media supplemented with 60 μg/ml G418 (Gibco-BRL).

Parasite protein extraction, SDS/PAGE and immunoblotting

Promastigotes were collected by centrifugation at 800 ***g*** for 10 min at 4 °C, washed with PBS and lysed directly (at 10^6 parasites/μl) into reducing sample buffer. Amastigotes were harvested from mouse footpad lesions 6–9 weeks post-infection. Proteins were separated by Tricine-SDS/PAGE [18] using pre-stained markers (Gibco-BRL), electroblotted on to Westran-PVDF membranes (0.2 μm; Schleicher and Schuell) and analysed following standard protocols [15]. For detection, secondary antibodies conjugated to horseradish peroxidase (Jackson Immunoresearch) were used in combination with the ECL Western Detection system (Amersham Pharmacia Biotech). Quantification of band intensities was carried out using the NIH Scion Image software package (National Institutes of Health).

Indirect immunofluorescence

Promastigotes were fixed and stained for DNA content and immunofluorescence as follows: PBS-washed cells were fixed for 30 min in 4% (w/v) paraformaldehyde in PBS, settled on to poly-L-lysine-coated slides for 30 min at room temperature, permeabilized for 2 min in 100% cold methanol and blocked for 2 h in PBS/3% (w/v) BSA. Primary and secondary antibody incubations were carried out for 1 h at room temperature in PBS/3% BSA, followed by washes in PBS for 30 min. Before mounting in Vectashield (Vecta Laboratories), slides were incubated in 20 μg/ml DAPI (4′,6′-diamidino-2-phenylindole) solution (Sigma) for 1 h. Fluorescent parasites were viewed using a Nikon Microphot FX epifluorescent microscope, images captured with a Photometrics CH350 CCD camera and data analysed via IPLab Spectrum software (Scanalytics).

A modification of the protocol of Lewis Carl et al. [19] was used to stain parasites with two rabbit polyclonal antibodies. Parasites were washed, fixed, permeabilized and blocked as described above. Slides were incubated with anti-BiP (immunoglobulin heavy-chain binding protein) antibody [20] at a 1:200 dilution for 1 h at room temperature, washed with PBS, and then incubated with fluorescein-conjugated goat anti-rabbit IgG (H + L) at a 1:2000 dilution followed by PBS washes. Slides were

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subsequently incubated with pre-immune rabbit serum at a 1:100 dilution for 1 h, washed with PBS and incubated with a 1:40 dilution of goat anti-rabbit Fab fragments (1.3 mg/ml; Jackson Immunoresearch) for 1 h. This incubation was followed by PBS washes and incubation with the anti-rHisD primary antibody at 1:50 dilution for 1 h. After further PBS washes, the slides were incubated with Cy3-conjugated goat anti-rabbit IgG at a 1:500 dilution for 1 h, stained with DAPI, washed and mounted.

Immunogold electron microscopy

Promastigotes were washed in PBS and incubated in 4% (w/v) paraformaldehyde/0.2 M Hepes, pH 7.2, for 5 min at room temperature followed by 1 h at 4 °C. The parasites were then collected, resuspended in 2% paraformaldehyde (w/v)/0.2 M Hepes, pH 7.2, and stored at 4 °C, prior to embedding in 1% (w/v) agarose and infiltration with 20% (w/v) polyvinylpyrrolidone in 1.8 M sucrose [21]. Ultrathin cryosections (80 nm) were cut, blocked with 1% (w/v) skimmed milk/0.5% (w/v) BSA in PBS for 20 min, labelled with anti-SHERP antibody (1:5000) for 1 h and goat anti-rabbit IgG coupled with Nanogold (1:50; Nanoprobes), followed by silver enhancement using the method of Danscher (see [22]). Staining and methylcellulose embedding were performed as described in [23].

In vivo cross-linking and membrane fractionation

Parasites (5×10^8) were washed, resuspended in 1 ml of PBS and incubated at 4 °C for 2 h with the thiol-cleavable homobifunctional cross-linker, dithiobis(succinimidyl propionate) (Pierce) at 4 mM, final concentration. In control experiments, solvent alone (DMSO) was added. The reaction was quenched by addition of 50 μl of 1 M Tris/HCl, pH 7.5, and incubation for 15 min at 4 °C. After further washing with PBS, the cells were mechanically lysed in 0.6 ml of lysis buffer (50 mM Hepes/KOH, pH 7.4/150 mM NaCl/2 mM EDTA, pH 8.0/10 μg/ml leupeptin/10 μg/ml aprotinin/0.5 mM PMSF/1 μM pepstatin) with 300 μm diam. glass beads (Sigma) at 4 °C. Undisrupted cells were cleared by centrifugation twice at 500 ***g*** for 10 min each at 4 °C. Membranes were isolated from the supernatant by ultracentrifugation at 100000 ***g*** for 1 h at 4 °C in a Beckman TLA 100.3 rotor. Cytosolic fractions were precipitated with 10% (w/v) trichloroacetic acid for 2 h at 4 °C and washed with 100% ethanol. Membrane and cytoplasmic proteins were resuspended in Laemmli sample buffer without reducing agents or sample buffer containing 5% (v/v) β-mercaptoethanol, boiled and size-separated by SDS/PAGE (10 and 15% gels) [15].

Actin-binding assay

Bovine muscle actin (Sigma) was dissolved in G-buffer (2 mM Tris/HCl, pH 8.0/0.2 mM ATP/0.2 mM $CaCl_2$/0.2 mM dithiothreitol) to give a final concentration of 1 mg/ml and cleared by centrifugation at 100000 ***g*** for 1 h at 4 °C, as described above. Actin was assembled at room temperature for 30 min in F-buffer (30 mM Tris/HCl, pH 8.0/0.2 mM ATP/0.1 M KCl/0.5 mM dithiothreitol/2 mM $MgCl_2$). G-actin and F-actin were mixed with recombinant SHERP protein or bovine pancreatic DNaseI (Sigma) in a 1:1 molar ratio and incubated for 15 min at 4 °C. Samples were separated further by 4–20% gradient PAGE (Bio-Rad) under non-denaturing conditions [24].

RESULTS

Analysis and conservation of the *Leishmania SHERP* genes

The *SHERP* genes are located on chromosome 23 of *L. major*, within the differentially expressed *LmcDNA16* family (Figure 1A). Also found within this locus are the stage-regulated *HASP* genes, which show extensive sequence identity at the nucleotide and amino acid levels. The *SHERP* genes, however, share only limited sequence similarity with the *HASP* genes, concentrated at the distal end of their 3′-untranslated regions (UTRs). These sequences have been implicated in stage-regulated expression at this locus ([12] and J. K. Keen, E. Lafuente-Duarte and D. F. Smith, unpublished work).

The two *L. major SHERP* genes are highly similar, showing 98.8% identity across the complete gene sequence and 100% identity within the ORF translated *in vivo* (see below). Gene duplications are quite common within the *Leishmania* genome [25], although the level of sequence identity between copies is only now becoming easily comparable, due to the *Leishmania* genome-sequencing project. Preliminary data indicate that *SHERP* copy number varies between strains of *L. major* ([13a] and D. F. Smith, unpublished work).

We demonstrate below that, as in the *HASP* genes [13], the first ORF downstream of the *SHERP* splice-acceptor site is translated *in vivo*. This ORF codes for a small 6.2 kDa protein with an acidic pI (4.6), that is very hydrophilic (Figure 1B), lacks transmembrane-spanning domains and, from its predicted secondary structure, has a high α-helical content with a helix-loop-helix motif (Figure 1C). The helical-wheel diagram in Figure 1(D) shows the amphiphilic character of SHERP with a clear separation of hydrophilic and hydrophobic residues on opposite sites of the α-helix, suggesting an involvement in protein–protein interactions. Such interactions do not seem to involve self-association, however, as there is no evidence for multimerization of native SHERP (results not shown). Similarly, phosphorylation experiments have failed to confirm that the two putative phosphorylation sites identified in motif searches are utilized *in vivo*.

The conservation of *SHERP* genes between Old- and New-World *Leishmania* species has been investigated by hybridizing blotted genomic DNA with a *SHERP*-specific probe including the complete ORF (results not shown). This analysis demonstrates that the *SHERP* genes are conserved across the *Leishmania* genus, although absent or of low identity in the evolutionarily distinct *L. braziliensis*.

Comparisons of the deduced *SHERP* ORFs in *L. major* and *L. donovani* show 79% identity and 100% similarity (T. Alce, unpublished work), although this is insufficient for cross-reactivity with the polyclonal anti-SHERP antibody raised for this study. In contrast, the same antibody does cross-react with *L. infantum* parasite lysate (results not shown).

BLAST searches do not reveal any recognizable homologues for *SHERP* in the existing databases. Considering the difficulties in finding distantly related genes using short target sequences, this is not surprising. Ongoing sequencing projects of closely related parasites like *Trypanosoma brucei* might, in the future, reveal functional *SHERP* homologues.

Cloning and expression of SHERP

The most 5′-proximal 171 bp *SHERP* ORF was amplified and cloned into the expression vectors pET33b(+) and pET15b, which both contain histidine-tags for affinity purification of the translated recombinant protein. Whereas the pET33b(+) con-

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Figure 1 Characteristics of SHERP deduced from the gene sequence

(**A**) Genomic organization of the *LmcDNA16* locus [12]. Genes *HASPA1*, *SHERP1*, *SHERP2*, *HASPB* and *HASPA2* are shown as boxes with the intergenic regions as connecting lines. Black shading represents nucleotide identity within the 3′ UTRs of *SHERP* and *HASP* genes; the sequences are otherwise unrelated. The positions of the *SHERP* ORFs translated *in vivo* are shown by arrows. Restriction sites: C, *Cla*I; X, *Xho*I. (**B**) Hydrophilicity analysis. Hydrophilicity plot of the translated 57 amino acid SHERP protein sequence using the Kyte Doolittle scale with a window size of 7 amino acids (MacVector sequence analysis software package, IBI). (**C**) PHD secondary-structure prediction for SHERP: AA, amino acid sequence; PHD_sec, PHD secondary structure prediction (H, helix; blank, loop); Rel_sec, reliability index for prediction (* symbolizes strong prediction). (**D**) α-Helical plot of amino acid residues 13–30 of SHERP: +, positively charged amino acids; −, negatively charged amino acids; ○, apolar residues.

struct was used to engineer SHERP with both N- and C-terminal His-tags, thereby increasing its size to 10.3 kDa (rSHERP-2xHis; Figure 2, lane 4), the pET15b construct encoded recombinant SHERP with only an N-terminal His-tag (8.3 kDa). These recombinant proteins, as well as the acidic native protein, migrate according to their molecular mass on Tricine-SDS/PAGE gels (Figure 2), in contrast to the HASPs, which migrate aberrantly [13]. The recombinant SHERPs were expressed, purified by Ni^{2+}-nitrilotriacetate affinity chromatography and used for polyclonal antibody production.

SHERP-overexpressing transgenic parasite lines were generated by cloning the 171 bp ORF into the shuttle vector pTEX [16] and transfection into wild-type *L. major* FVI and the double-knockout *LmcDNA16*-null mutant line, *ΔcDNA16::HYG/ΔcDNA16::PAC*. Six independent clones of FVI [*pTEX NEO SHERP*^{His}] and nine independent clones of *ΔcDNA16::HYG/ΔcDNA16::PAC* [*pTEX NEO SHERP*^{His}] were identified by PCR and genomic Southern blotting (results not shown). To facilitate purification of SHERP from transfected parasites, the episomally encoded SHERP carries a C-terminal

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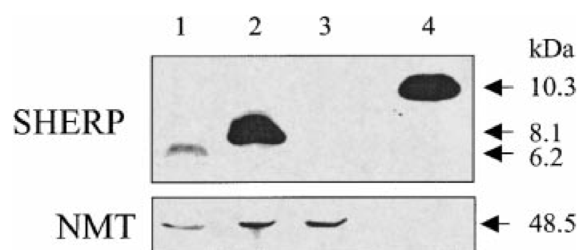

Figure 2 Characterization of SHERP-overexpressing transgenic parasites *L. major* [*pTEX NEO SHERP^{His}*]

Parasite lysate (10^7 cells/lane) and purified rSHERP-2xHis protein were separated on a Tricine-SDS/PAGE gel and blotted. The immunoblot was incubated with anti-SHERP antibody (1:5000) and secondary horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:60000) and detected using ECL. The same blot was then incubated with an antibody detecting the constitutively expressed N-myristoyltransferase (anti-NMT antibody, 1:1000) followed by horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:60000) and ECL detection. Lane 1, metacyclic *L. major* FVI parasites; lane 2, *L. major* [*pTEX NEO SHERP^{His}*] on day 3 of culture; lane 3, *L. major* [*pTEX NEO*] on day 3 of culture; lane 4, 40 ng of rHisD protein. Size standards are given on the right.

His-tag that increases its size to 8.1 kDa (Figure 2, lane 2). This protein is constitutively expressed in pTEX because only the *SHERP* ORF and not the UTRs is part of this plasmid. Wild-type parasites carrying the pTEX vector alone show no SHERP expression in this life-cycle stage (Figure 2, lane 3). These transgenic parasite lines were used in the immunocytochemical analysis and functional studies described below.

Stage-specific expression of SHERP

Previous analyses of steady-state RNA levels have shown that the RNA transcripts derived from the polycistronically transcribed *LmcDNA16* locus are differentially expressed [9,12]. *HASPB* and *HASPA1* mRNAs are detected only in metacyclic and amastigote stages, whereas *HASPA2* mRNA is present at a low level in procyclics, more abundant in metacyclics but absent from amastigotes. Flinn and Smith [12] also detected a 1.6 kb transcript, abundant in metacyclics, that was derived from the *SHERP* genes (then named the D genes). Here, we confirm the up-regulation of *SHERP* transcripts in metacyclics (Figure 3A) and also detect varying levels in procyclics and amastigotes. To determine the extent of metacyclic contamination in the procyclic population used for RNA extraction, the same RNA blot was also hybridized with a β-tubulin probe, which detects a constitutive 2.2 kb transcript and additional RNAs of 3.2 kb (stabilized in metacyclics) and 2.8 kb (up-regulated in amastigotes) [26]. This confirms low-level contamination of metacyclic transcripts in the procyclic RNA sample. These data support a model in

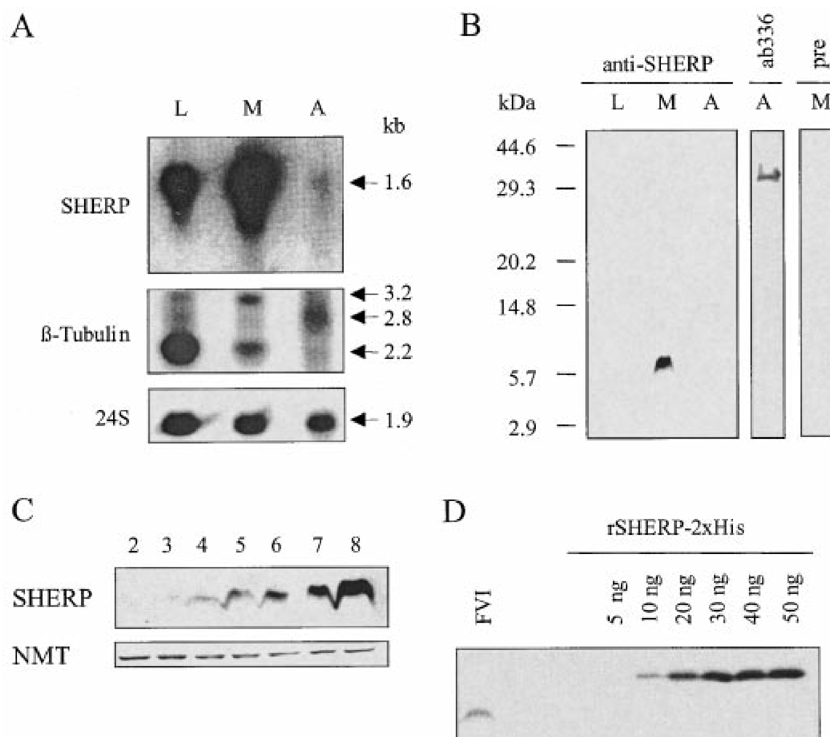

Figure 3 Expression pattern and abundance of SHERP

(**A**) Northern-blot analysis. Total RNAs extracted from procyclics (L), metacyclics (M) and amastigotes (A) were denatured, size-separated on 1.2% formaldehyde/agarose gel and blotted prior to hybridization with a 602 bp SHERP-specific probe, including the ORF. The blot was re-hybridized with a stage-regulated probe (β-tubulin) and a non-developmentally regulated 24 S rRNA probe as loading control. Transcript sizes (kb) are indicated on the right of each blot. (**B**) Immunoblot detection of native SHERP in procyclics, metacyclics and amastigotes. Parasite protein lysates (10^7 cells/lane) were separated by Tricine-SDS/PAGE and blotted, prior to incubation with anti-SHERP antibody (1:5000) and detection by ECL. To confirm the presence of intact amastigote proteins, the blot was incubated subsequently with antibody 336 (1:1500) that detects HASPB expression in amastigotes. A similar blot prepared from the same metacyclic protein lysate was incubated with pre-immune sera (pre; 1:4000). Protein size markers are shown on the left (kDa). (**C**) SHERP expression during parasite differentiation. Protein lysates (8×10^6 cells/lane) from parasites cultured over a time course (shown at the top, in days) were separated by Tricine-SDS/PAGE and blotted prior to incubation with anti-SHERP antibody (1:5000) and detection by ECL. Detection of N-myristoyltransferase (NMT) was used as a loading control. (**D**) Evaluation of SHERP abundance. Total metacyclic parasite lysate (10^7 parasites) and purified rSHERP-2xHis protein were separated by Tricine-SDS/PAGE, blotted and probed with anti-SHERP antibody (1:5000) as in (**C**). Band intensities were compared using the NIH Scion Image software package.

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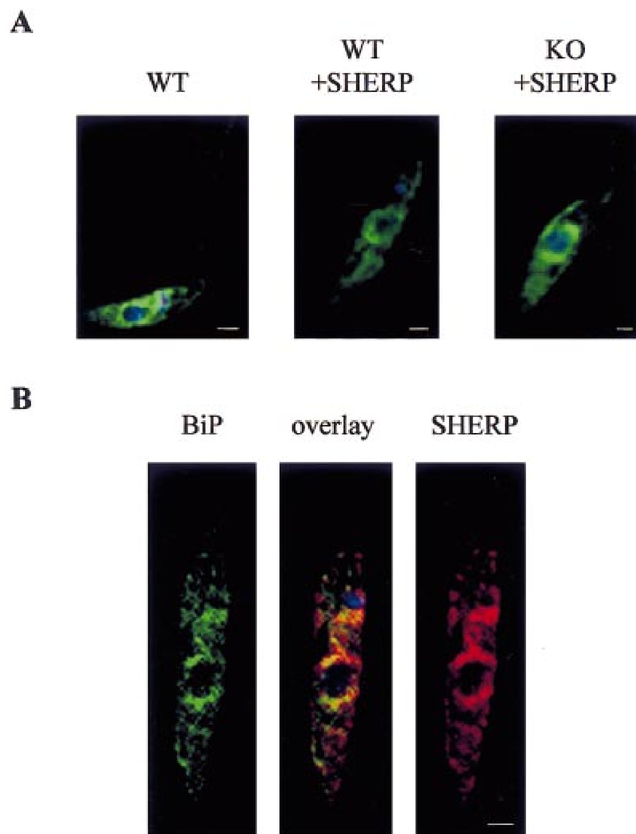

Figure 4 Indirect immunofluorescence detection of SHERP expression in wild-type and overexpressing *L. major* promastigotes

(**A**) SHERP expression in wild-type and overexpressing parasites. *L. major* FVI wild-type metacyclics (cultured for 8 days; WT), [*pTEX NEO SHERP*^{His}] transfectants (cultured for 4 days; WT + SHERP) and Δ*cDNA16::HYG*/Δ*cDNA16::PAC* [*pTEX NEO SHERP*^{His}] transfectants (cultured for 4 days; KO + SHERP) were stained with anti-rHisD antibody, which specifically recognizes SHERP in these lines (shown in green). Nuclear and kinetoplast DNAs are shown in blue. (**B**) Co-localization of BiP and SHERP in procyclic [*pTEX NEO SHERP*^{His}] transfectants, carried out as described in the text. Nuclear and kinetoplast DNAs are shown in blue. Scale bars, 1 μm.

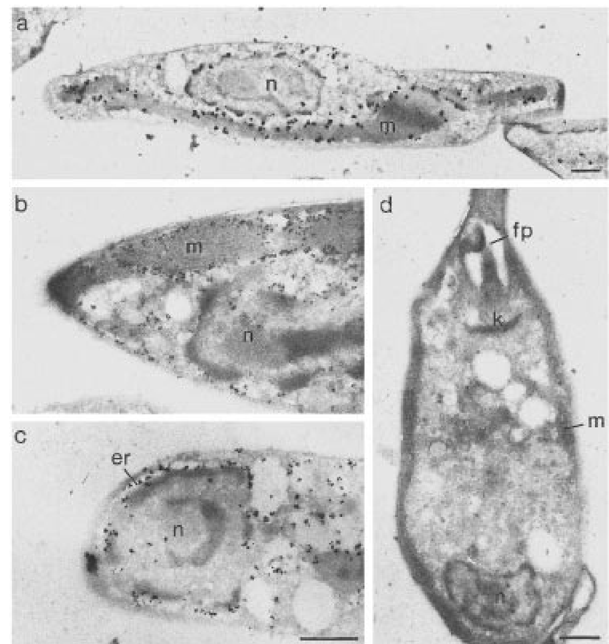

Figure 5 SHERP localization in *L. major* metacyclics by immunogold electron microscopy

(**a**), (**b**) and (**c**) show ultrathin cryosections of wild-type *L. major* FVI metacyclic parasites labelled with anti-SHERP antibody and Nanogold-conjugated goat anti-rabbit IgG, after silver enhancement. (**a**) and (**b**) show SHERP localization to the outer mitochondrial membrane; (**c**) shows labelling around the nucleus, indicative of localization to the ER; (**d**) is a negative control showing metacyclic Δ*cDNA16::HYG*/Δ*cDNA16::PAC* parasites treated identically to the wild-type parasites in (**a**–**c**). er, ER; fp, flagellar pocket; k, kinetoplast; m, mitochondrion; n, nucleus. Scale bars, 0.5 μm.

which the stabilization of *SHERP* transcripts correlates with parasite differentiation from procyclics to metacyclics (J. K. Keen, E. Lafuente-Duarte and D. F. Smith, unpublished work).

SHERP expression was monitored by probing immunoblots of parasite lysates from different life-cycle stages. As shown in Figure 3(B), the 6.2 kDa SHERP protein is only detectable in metacyclics, and not in either of the replicative parasite stages. HASP antibody 336 was used to detect HASPB (migrating at 36–38 kDa) in the lesion amastigotes, thus confirming the integrity of proteins isolated from these parasite stages. In a time course of SHERP expression during differentiation (Figure 3C), no protein was detectable in a 2 day culture but a low level of expression was observed after 3 days, with an increase in abundance as the parasites underwent metacyclogenesis. Expression of the constitutively expressed N-myristoyltransferase (M. Menon, unpublished work) was used to monitor equivalent protein loading on these blots.

These results demonstrate clearly the predominant expression of SHERP in metacyclic parasites, although *SHERP* mRNA is detectable to some degree in all life-cycle stages. Thus SHERP expression may be regulated, at least in part, by a translational control mechanism. These observations concur with previous studies demonstrating different control points for post-transcriptional regulation in *Leishmania*, including RNA stability [27] and translational regulation [28]. The abundance of SHERP in metacyclics was determined relative to the recombinant protein, rSHERP-2xHis (Figure 3D). Different parasite numbers were loaded relative to rSHERP in independent experiments and NIH Scion Image analysis of band intensities gave an approximate estimate of 100000 copies/metacyclic parasite. This is relatively high when compared with the two most abundant *Leishmania* surface proteins characterized to date: gp63 (or leishmanolysin), the main surface protease of *L. major*, at $(3\text{–}5)\times10^5$ molecules/promastigote [29] and KMP11 of *L. donovani* at 1–2 million molecules/parasite [30].

Localization of SHERP in wild-type and genetically modified parasite lines

The SHERP protein was localized in wild-type metacyclics and SHERP overexpressing transgenics by indirect immunofluorescence and immunogold-labelling assays (Figures 4 and 5, respectively). No staining was detected in freshly isolated lesion amastigotes or in pure procyclic cultures of wild-type FVI or FVI [*pTEX NEO*] parasites (results not shown). In wild-type metacyclics, SHERP stain concentrates as a perinuclear ring around the unstained nucleus and spreads outwards in interconnecting band-like structures towards the kinetoplast on one side and towards the posterior tip on the other (Figure 4A). This staining

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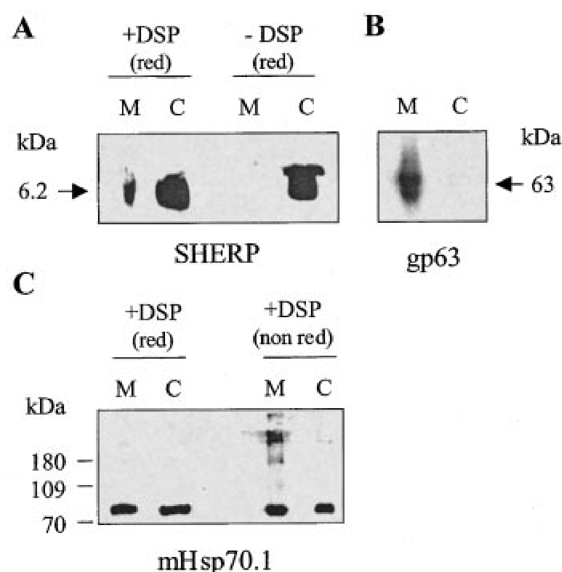

Figure 6 Membrane association of SHERP after cross-linking *in vivo*

(**A**) SHERP detection in immunoblotted membrane (M) or cytoplasmic (C) fractions from cross-linked [+DSP, dithiobis(succinimidyl propionate)] versus non-crosslinked (-DSP) *L. major* wild-type FVI parasites, using anti-SHERP antibody (1:5000) and horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:60000). (**B**) gp63 detection only in the immunoblotted membrane fraction analysed in (**A**), using anti-gp63 antibody (1:2000), demonstrates the lack of membrane contamination in the cytoplasmic fraction. (**C**) Cross-linking control: immunoblot showing the membrane fractions analysed in (**A**) labelled with pX70.1A antibody (1:2000 [32]) that detects mitochondrial hsp70 in *L. major.* High-molecular-mass cross-linking products were detected in the sample lacking reducing agents. red, reducing sample buffer containing β-mercaptoethanol; non red, non-reducing sample buffer. Molecular masses are indicated beside each panel (kDa).

pattern is unaffected by different permeabilization methods (results not shown). A comparable staining pattern is also evident in the two SHERP-overexpressing parasite lines (Figure 4A). These parasites are advantageous for these experiments as stage-regulated SHERP expression is lost and localization can therefore be carried out in the larger procyclic promastigotes.

The perinuclear ring localization for SHERP suggested a possible association with the ER. Therefore, SHERP-over-expressing parasites were used to co-localize SHERP with a well-characterized ER luminal marker, BiP [20]. As shown in Figure 4(B), the BiP and SHERP labellings partially co-localize, especially around the nucleus and towards the kinetoplast. In other areas of the parasite, however, the antibody localization does not overlap. The ER is a continuous network, believed to be subdivided into specialized areas. Thus SHERP and BiP might localize to different areas of this network or SHERP might localize additionally to other internal structures. Control experiments using mitochondrial-matrix-localized mHsp70-specific and BiP-specific rabbit antisera together showed no co-localization, confirming the specificity of our co-localization procedure (results not shown).

Further examination of the immunofluorescence images (Figure 4) suggested that the band-like staining observed distinct from the ER regions might be mitochondrial in origin. This was confirmed by immunogold electron microscopy. In some cryosections, the mitochondrial membrane was strongly labelled with the SHERP antibody (Figures 5a and 5b), whereas in others the perinuclear stain was evident, as were some gold particles in the cytoplasm (Figure 5c). Whether this cytoplasmic stain represents gold particles associating with collapsed ER tubular structures, cytoplasm-localized SHERP or is simply an artifact of the preparation method used cannot be assessed directly.

Membrane association of SHERP

In order to confirm biochemically the SHERP membrane association observed by electron microscopy, cell fractionation of wild-type metacyclics was performed as described. Membrane and cytosolic fractions were acid-precipitated before SDS/PAGE and immunoblotting. By this analysis, SHERP is found exclusively in the cytosolic fraction (Figure 6A). However, if SHERP's association with membranes is by weak interactions, these might not be retained after cell disruption. Thus in order to stabilize putative interactions *in vivo*, parasites were incubated with the homobifunctional cross-linker dithiobis(succinimidyl propionate), which is described to be membrane permeable and has been used to cross-link proteins *in vivo* [31]. After cross-linking *in vivo* for 2 h, parasites were cell-fractionated as before. Immunoblots of these fractions, after separation by SDS/PAGE, show partial redistribution of SHERP to the membrane fraction (Figure 6A). The fact that only a proportion of the SHERP population is redistributed in this way might be due to the ineffiency of this *in vivo* cross-linking method in *Leishmania* and/or a cytosolic pool of SHERP that is not membrane-associated. The electron-microscopic data support the first option more strongly, suggesting that most SHERP is associated with internal membranes in metacyclic parasites. Investigating the distribution between membrane and cytosolic fractions of other proteins such as the plasma-membrane protein HASPB and the mitochondrial protein Hsp70.1, before and after cross-linking, showed no significant change (results not shown). To confirm that the cellular-fractionation method used is robust, membrane and cytosolic fractions were immunoblotted and probed with anti-gp63 antibody, that recognizes the glycosylphosphatidylinositol-anchored zinc metalloproteinase of *Leishmania*. gp63 fractionates exclusively in the membrane component, as expected. By contrast, an immunoblot probed with an antibody against the mitochondrial-matrix protein Hsp70.1 [32], a chaperone involved in protein import and therefore associated with protein components of the inner mitochondrial membrane [33], detected an equal distribution between each fraction (Figure 6C). The effectiveness of the cross-linking *in vivo* is evident with the detection of additional high-molecular-mass bands under non-reducing conditions (Figure 6C). Under identical conditions, no additional bands indicative of interacting proteins were detectable on SHERP blots (results not shown). However, given the small size of this protein, it is likely that the epitopes recognized by the polyclonal rabbit serum would be masked during interaction with one or more possibly larger protein partners.

These data support the proposed association of SHERP with internal membranes. Given its hydrophilicity and lack of membrane-spanning domains or anchors, this assocation is likely to be mediated by another peripheral or internal membrane protein specific for the ER and mitochondrial outer membrane. The lack of a typical N-terminal signal sequence for protein import suggests that SHERP does not translocate membranes but remains in association with the cytosolic face. However, the presence of thus-far uncharacterized internal targeting motifs cannot be ruled out.

SHERP localization is independent of an intact cytoskeleton

As one approach to determining a function for SHERP, we screened existing protein databases for low-molecular-mass

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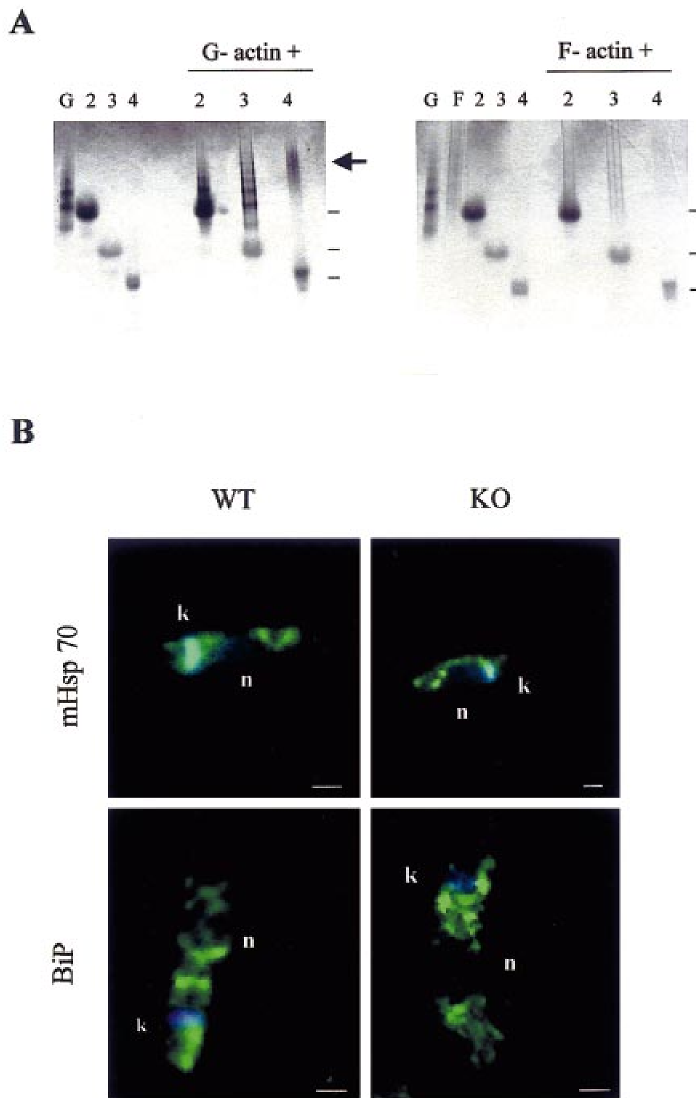

Figure 7 Actin-binding assay and preservation of ER and mitochondrial morphology in *LmcDNA16*-null parasites

(**A**) G-actin (bovine) and F-actin were incubated with rSHERP-2xHis (2), rSHERP-His (3) and DNaseI (4) for 15 min on ice before separation on 4–20% gradient gels under non-denaturing conditions and staining with Coomassie Brilliant Blue. Markers on the right of each panel indicate the migration positions of rSHERP-2xHis, rSHERP-His and DNaseI (from top to bottom). Under these conditions, polyhistidine tags cause protein aggregation [37]. The arrow indicates a shift in mobility as a result of the association of DNaseI with G-actin (which shows multimerization, probably as a result of product impurities). (**B**) Indirect immunofluorescence analysis of wild-type *L. major* FVI metacyclics (WT) and Δ*cDNA16::HYG*/Δ*cDNA16::PAC* metacyclics (KO) stained with pX70.1A antibody (1:50) and anti-BiP antibody (1:100). n, nucleus; k, kinetoplast (shown in blue).

proteins (< 10 kDa) of acidic pI. This identified mouse thymosin β4 (accession no. CAA34187), a predominantly hydrophilic, 5 kDa protein of pI 5.1, which has a large α-helical content. Thymosin β4 is expressed in different vertebrate cell types, possesses an actin-binding motif and functions as a G-actin-sequestering protein [34]. Comparison of the SHERP and thymosin β4 amino acid sequences showed 49% similarity but only 16% identity, and this did not include the actin-binding region. However, such motifs are not always conserved: the apicomplexan parasite, *Toxoplasma*, has an actin-binding protein, toxofilin, that lacks a classical actin-binding signal [35].

Given this information, we investigated whether SHERP can bind either G- or F-actin and might therefore have a role in interaction with the cytoskeleton and organellar morphology. In native PAGE experiments, recombinant SHERP is unable to bind G- or F-actin (Figure 7A). A shift in migration was only observed with DNaseI (31.4 kDa), a known G-actin-binding protein [36].

We further investigated whether SHERP is required for the preservation of organelle morphology in *Leishmania*. Wild-type and *LmcDNA16*-null parasites were fixed and incubated with antibodies to detect mHsp70, the mitochondrial-matrix marker, and BiP, the ER lumen marker (Figure 7B). No difference in mitochondrial or ER morphology could be detected in procyclics and metacyclics of the *LmcDNA16* double-knockout parasites (lacking *SHERP*) relative to wild-type parasites. Thus it is unlikely that SHERP plays a direct role in organelle morphology or cytoskeletal attachment.

DISCUSSION

The *SHERP* genes are highly conserved within the *Leishmania* genus although no homologues have yet been found in other species. There are six putative translational ORFs (not all in frame) within each *SHERP* gene, as delimited by the 5′ acceptor site for *trans*-splicing and the 3′ heterogeneous region of polyadenylation. Here, we demonstrate that the first ORF downstream of the splice-acceptor site codes for a 57 amino acid protein that is expressed *in vivo*. Previous data [38,39] have suggested that the fourth ORF, towards the 3′-end of the gene, codes for a 15.4 kDa leucine-zipper-containing protein. However, a polyclonal antibody raised against this fourth ORF expressed as a recombinant protein in *E. coli* failed to detect a parasite protein in our hands (results not shown). As there are only a few examples of more than one ORF translated per mRNA [40], we consider it unlikely that other ORFs in *SHERP* are utilized *in vivo*.

The *SHERP* transcript is highly up-regulated in infective metacyclic parasites [12,38,41], in which it represents the dominant transcript relative to other differentially expressed mRNAs. Although *SHERP* mRNA is detectable in other parasite stages, the SHERP protein is expressed exclusively with a high copy number in metacyclics and is not detectable in replicative parasites. Metacyclic parasites are pre-adapted for host-cell entry, with extensive modification of their surface glycoconjugate coat, the best-characterized biochemical change that correlates with metacyclogenesis. However, it is also known that translation in general is down-regulated in these cells [41a] and increased uptake of some nutrients has been proposed [42].

The only other protein predominantly expressed in metacyclics that has been characterized to date is meta 1 [10], an 11.5 kDa protein of unknown function that localizes to intracellular vesicles close to the parasite flagellar pocket. The *meta* 1 gene is essential for viability but its overexpression correlates with increased virulence in transgenic parasites [11]. Unlike meta 1, SHERP is localized on the ER as well as the outer mitochondrial membrane, whereas the *LmcDNA16*-null mutants (lacking SHERP function) are viable and at least as infective as wild-type parasites (P. G. McKean, P. W. Denny, E. Knuepfer and D. F. Smith, unpublished work). However, these transgenics have not yet been tested for their ability to undergo metacyclogenesis in the sandfly vector.

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The attachment of SHERP to membranes, presumably by weak interactions of the amphiphilic α-helices with peripheral or integral membrane protein(s), is unusual. A similar type of attachment to the ER membrane is proposed for yeast acetyl-CoA carboxylase [43] which, like SHERP, is hydrophilic and predicted to attach to an integral ER membrane protein via its C-terminus. However, localization to both ER and mitochondrial membranes is rare and, to our knowledge, only described to date for integral membrane proteins, such as cytochrome b_5 [44], NADH-cytochrome b_5 reductase [45] and Bcl-2 [46]. Mitochondria and the ER do have close contact sites that are implicated in the control of cytosolic Ca^{2+} levels [47] or, in the case of the mitochondrially associated membranes, in phospholipid translocation between the ER and the mitochondrion [48]. It is unclear whether SHERP plays a role in any of these processes, however.

An alternative function for SHERP could be as a component of a protein-import system in these organelles. Although the components of these complexes are not obviously related, they share common features, including the presence of small membrane-bound subunits, e.g. the 6 kDa yeast Tom5 protein, involved in target-protein entry into the mitochondrial translocation pore [49], and the 9 kDa yeast Sss1p protein, part of the ER translocation machinery [50]. Differences in the ER protein-import mechanism have been demonstrated between trypanosomatids and mammalian cells [51], whereas the mechanism of mitochondrial import is probably conserved [52]. In addition to protein import, trypanosomatids also transport tRNAs (encoded in the nuclear genome) into their mitochondria [53]. This is unusual when compared with many other eukaryotes, in which tRNA genes are found in the mitochondrial genome. We can neither confirm nor dispute a role for SHERP in these processes.

Mitochondria and ER structures have been shown to co-localize with both microtubules [54] and actin cables [55]. Under our assay conditions, SHERP does not bind actin directly. Additional analyses of ER and mitochondrial morphology in transgenic parasites lacking SHERP function show no detectable differences, suggesting that SHERP is not involved in cytoskeletal processes. In summary, although the function of SHERP remains unclear, we speculate that SHERP expression may be a specific requirement for organellar function during *Leishmania* differentiation, an essential process for vector transmission.

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